BARD Final Report US-4551-12

Environmental fate of antiepileptic drugs and their metabolites: Biodegradation, complexation, and photodegradation

Borch, T. Colorado State University CO

Hadar, Y. The Hebrew University of Jerusalem

Polubesova, T. The Hebrew University of Jerusalem

Project award year: 2012

Three year research project

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Abstract

Many pharmaceutical compounds are active at very low doses, and a portion of them regularly enters municipal sewage systems and wastewater-treatment plants following use, where they often do not fully degrade. Two such compounds, CBZ and LTG, have been detected in wastewater effluents, surface waters, drinking water, and irrigation water, where they pose a risk to the environment and the food supply. These compounds are expected to interact with organic matter in the environment, but little is known about the effect of such interactions on their environmental fate and transport. The original objectives of our research, as defined in the approved proposal, were to:

- Determine the rates, mechanisms and products of photodegradation of LTG, CBZ and selected metabolites in waters exposed to near UV light, and the influence of DOM type and binding processes on photodegradation.
- Determine the potential and pathways for biodegradation of LTG, CBZ and selected metabolites using a white rot fungus (*Pleurotus ostreatus*) and ADP, and reveal the effect of DOM complexation on these processes.
- Reveal the major mechanisms of binding of LTG, CBZ and selected metabolites to DOM and soil in the presence of DOM, and evaluate the effect of this binding on their photodegradation and/or biodegradation.

We determined that LTG undergoes relatively slow photodegradation when exposed to UV light, and that pH affects each of LTG's ability to absorb UV light, the efficiency of the resulting reaction, and the identities of LTG's photoproducts ($t_{½}$ = 230 to 500 h during summer at latitude 40 °N). We observed that LTG's photodegradation is enhanced in the presence of DOM, and hypothesized that LTG undergoes direct reactions with DOM components through nucleophilic substitution reactions. In combination, these data suggest that LTG's fate and transport in surface waters are controlled by environmental conditions that vary with time and location, potentially affecting the environment and irrigation waters.

We determined that *P. ostreatus* grows faster in a rich liquid medium (glucose peptone) than on a natural lignocellulosic substrate (cotton stalks) under SSF conditions, but that the overall CBZ removal rate was similar in both media. Different and more varied transformation products formed in the solid state culture, and we hypothesized that CBZ degradation would proceed further when *P. ostreatus* and the enzymatic profile were tuned to lignin degradation. We observed ^{14}C-CO_2 release when ^{14}C-carbonyl-labeled CBZ was used as the substrate in the solid state culture (17.4% of the initial radioactivity after 63 days of incubation), but could not conclude that mineralization had occurred. In comparison, we determined that LTG does not degrade in agricultural soils irrigated with treated wastewater, but that *P. ostreatus* removes up to 70% of LTG in a glucose peptone medium. We detected various metabolites, including N-oxides and glycosides, but are still working to determine the degradation pathway. In combination, these data suggest that *P. ostreatus* could be an innovative and effective tool for CBZ and LTG remediation in the environment and in wastewater used for irrigation.

In batch experiments, we determined that the sorption of LTG, CBZ and selected metabolites to agricultural soils was governed mainly by SOM levels. In lysimeter experiments, we also observed LTG and CBZ accumulation in top soil layers enriched with organic matter. However, we detected CBZ and one of its metabolites in rain-fed wheat previously irrigated with treated wastewater, suggesting that their sorption was reversible, and indicating the potential for plant uptake and leaching. Finally, we used macroscale analyses (including adsorption/desorption trials and resin-based separations) with molecular-level characterization by FT-ICR MS to demonstrate the adsorptive fractionation of DOM from composted biosolids by mineral soil. This suggests that changes in soil and organic matter types will influence the extent of LTG and CBZ sorption to agricultural soils, as well as the potential for plant uptake and leaching.

Summary Sheet

Publication Summary

PubType	IS only	Joint	US only
Reviewed	1	2	1
Submitted	0	1	0

Training Summary

Trainee Type	Last Name	First Name	Institution	Country
Ph.D. Student	Golan	Naama	The Hebrew University of Jerusalem	Israel
M.Sc. Student	Paz	Anat	The Hebrew University of Jerusalem	Israel
M.Sc. Student	Salton	Orit	The Hebrew University of Jerusalem	Israel
M.Sc. Student	Tadmor	Galit	The Hebrew University of Jerusalem	Israel
Ph.D. Student	Troyer	Lyndsay	Colorado State University	USA
Ph.D. Student	Young	Robert	Colorado State University	USA

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Contribution of collaboration

All project objectives were promoted as a result of the cooperation, through meetings, correspondence, shared experiments, and co-authorships involving the Israeli and US research groups. These activities included planning sessions, joint trainings, sample and data analysis, and critical reviews of everyone's work and communication of results. The combined expertise of the Israeli and US research groups permitted thoughtful and comprehensive discussions about hypotheses and results. In addition, activities like joint training at the National High Magnetic Field Laboratory enabled us to analyze complex datasets with a common knowledge base. The success of our collaboration is reflected in our achievements. We found the cooperation valuable, and intend to continue working together.

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Achievements

Among emerging contaminants, pharmaceutical compounds raise special concerns because they are designed to have biological effects at low concentrations, they act on biological systems that often are conserved across many species, they can produce toxic idiosyncratic reactions in selected individuals, and their mixtures can produce additional adverse effects.[1, 2] We selected CBZ and LTG as model pharmaceutical compounds, but CBZ and LTG also raise specific concerns because they appear to be recalcitrant, and they act upon voltage-gated sodium channels, which form the basis of electrical excitability in most vertebrate and invertebrate species.[3, 4] CBZ, in particular, has been described as the most frequently studied and detected pharmaceutical compound in freshwater ecosystems in North America and Europe, and third in Asia.[5] In addition, CBZ is among the most frequently detected pharmaceutical compounds in drinking water, and plant uptake has been observed.[6, 7] Similarly, LTG recently has been detected in wastewater, groundwater, surface water, and drinking water.[8, 9] Furthermore, in one study that sought to prioritize the environmental risk of the top 200 prescription drugs from 2009, LTG was one of only 13 pharmaceutical compounds that appeared in the top 20 for at least 6 of the 12 toxic endpoints considered.[10]

The original objectives and most significant findings of our research are set forth in the abstract. The main scientific achievements of our research are:

- We gained new information about the environmental fate and transport of LTG by studying its photodegradation in surface waters, its biodegradation in agricultural soils and by white-rot fungi, and its sorption to agricultural soils. We determined degradation kinetics and byproducts, and identified various factors that would influence LTG’s presence and persistence in soils and waters.
- We gained new information about the biodegradation of CBZ by white-rot fungi, including information on the enzymatic systems involved, the conditions necessary to produce more complete degradation (possibly to mineralization), and the degradation products. We also gained new information about the sorption of CBZ to agricultural soils, and the potential for leaching and plant uptake.
- We gained new information about the adsorptive fractionation of organic matter by mineral soil, and the role of SOM in LTG and CBZ sorption by agricultural soils.

Our achievements are significant because they provide new details on the environmental fate and transport of two important emerging contaminants, LTG and CBZ, and because they provide insights into the transformation processes that govern other pharmaceutical compounds and environmental contaminants. We have shown that white-rot fungi are effective at removing pharmaceutical compounds that are commonly considered recalcitrant. This could have important implications for water treatment and remediation. We have shown that water conditions can have a significant effect on photodegradation in surface waters, and have proposed reaction mechanisms to explain this effect. This could have important implications for contaminant modeling and contaminant prioritization in local environments. We have identified transformation products, some of which are known or suspected to be toxic, and the mechanisms that produced them. This could help in determining transformation products from other contaminants, and could help in identifying targets for soil and water analysis. Finally, we have demonstrated the adsorptive fractionation of organic matter by a mineral soil. This could help in understanding carbon cycling, organic matter mobility, the relative persistence of individual SOM components, and the reactivity of organic matter towards pollutants, nutrients, and soil particles.

With respect to agriculture in particular, we determined that SOM levels govern the sorption of LTG and CBZ by agricultural soils, and observed reversible sorption, suggesting the potential for leaching or plant uptake over time. We also demonstrated that the type of soil and organic matter will affect both sorption and desorption in agricultural soils. This information could have special significance when composted biosolids or treated wastewater are applied to soils, especially considering the potential for plant uptake. The data on biodegradation by *P. ostreatus* suggests the possibility of effective remediation strategies for biosolids and treated wastewater before they are applied to soils. Finally, the data on photodegradation might help to predict the persistence of contaminants and their degradation products in surface waters used for irrigation.

Our achievements have fulfilled most of our original research objectives. We have not revealed the effect of DOM complexation on biodegradation by *P. ostreatus*, but the results in liquid and solid state media and the data on enzyme involvement provide the framework for continuing this study. Similarly, we did not demonstrate the effect of DOM type and binding processes on LTG photodegradation, but we did observe enhanced photodegradation in the presence of DOM, and we identified a mechanism that could demonstrate direct reactions between LTG and DOM during photodegradation. In the upcoming months, we intend to use FT-ICR MS to look for the byproducts of these reactions and demonstrate a previously unreported photoreaction involving DOM in surface waters. After considering our objectives and achievements, we believe that this project was a success.

References

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List of Abbreviations (in alphabetical order)

ADP	atrazine-degrading Pseudomonas
CBZ	carbamazepine
CYP-450	cytochrome P450 monooxygenase
DOM	dissolved organic matter
FT-ICR MS	Fourier transform ion cyclotron resonance mass spectrometry
LC-MS	liquid chromatography-mass spectrometry
LTG	lamotrigine
SSF	solid-state fermentation
SOM	soil organic matter
UV	ultraviolet

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Changes to original research plan

We fulfilled most of our original research objectives, as indicated in the description of our achievements. We prioritized tasks according to the data we received, and did not complete every task described in the work plan in our original proposal (e.g., photodegradation studies of biodegradation metabolites, and biodegradation studies of photoproducts). Changes to the original research plan follow:

Objective 1

When studying photodegradation, we focused our initial efforts on LTG because previous studies had examined the direct and indirect photodegradation of CBZ, as explained in our original proposal. The byproducts of LTG photodegradation and biodegradation by *Pleurotus ostreatus* were completely different, so we focused our efforts on explaining the effect of interactions between LTG and DOM on photodegradation, rather than on examining the photodegradation of *P. ostreatus* byproducts. One mechanism of LTG photodegradation suggested the possibility of direct reactions, other than energy transfer reactions, between LTG and DOM components. Such reactions have not been reported previously, and we are continuing to pursue this possibility for a separate manuscript (in preparation).

Objective 2

10,11-epoxycarbamazepine is a known photoproduct of lamotrigine, and a metabolite during biodegradation by *P. ostreatus*. Accordingly, the CBZ biodegradation studies did consider at least one CBZ photoproduct. However, the byproducts of LTG photodegradation and biodegradation by *Pleurotus ostreatus* were completely different, and no biodegradation was observed when LTG was exposed to agricultural soils, even when the media was enriched with chloramphenicol or acidified or when LTG served as the sole N source. It is possible that sorption to agricultural soils inhibited LTG biodegradation by microbes. Thus, we conducted similar experiments in soil suspensions, where sorption plays little role. We elected to focus our efforts on LTG biodegradation by *P. ostreatus*, which has a wide variety of oxidizing enzymes and which was successful in degrading CBZ.

Objective 3

We determined the sorption affinities of LTG, CBZ and selected metabolites to agricultural soils in batch and lysimeter experiments, and the selective adsorption of DOM from composted biosolids to mineral soils. We evaluated uptake of CBZ and one of its metabolites by rain-fed wheat previously irrigated with treated wastewater. We also have examined the selective adsorption of resin-based fractions to mineral soils, which may lead to an additional publication. However, we did not employ the equilibrium dialysis system described in our original proposal during these studies.

Publications for Project US-4551-12

Status	Type	Authors	Title	Journal	Vol:pg Year	Coun
Published	Reviewed	*Grossberger, A., Y. Hadar, T. Borch, and B. Chefetz.*	Biodegradability of pharmaceutical compounds in agricultural soils irrigated with treated wastewater	***Environmental Pollution***	185 : 168-177 2014	Joint
Published	Reviewed	*Young, Robert B., Benny Chefetz, Aiju Liu, Yury Desyaterik, and Thomas Borch*	Direct photodegradation of lamotrigine (an antiepileptic) in simulated sunlight - pH influenced rates and products	***Environmental Science-Processes & Impacts***	16 : 848-857 2014	US only
Published	Reviewed	*Golan-Rozen, N., B. Seiwert, C. Riemenschneider, T. Reemtsma, B. Chefetz, and Y. Hadar*	Transformation Pathways of the Recalcitrant Pharmaceutical Compound Carbamazepine by the White-Rot Fungus Pleurotus ostreatus: Effects of Growth Conditions	***Environmental Science & Technology***	49 : 12351-12362 2015	IS only
Published	Reviewed	*Paz, Anat, Galit Tadmor, Tomer Malchi, Jens Blotevogel, Thomas Borch, Tamara Polubesova, and Benny Chefetz*	Fate of carbamazepine, its metabolites, and lamotrigine in soils irrigated with reclaimed wastewater: Sorption, leaching and plant uptake	***Chemosphere***	160 : 22–29 2016	Joint
Submitted	Reviewed	*Avneri-Katz, Shani, Robert B. Young, Amy M. McKenna, Huan Chen, Yuri E. Corilo, Tamara Polubesova, Thomas Borch, and Benny Chefetz*	Adsorptive fractionation of dissolved organic matter (DOM) by mineral soil: Macroscale approach and molecular-level insight	***Organic Geochemistry***	: 2016	Joint

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APPENDIX

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Cover Page

BARD Project Number: US-4551-12

Date of Submission of the Report: June 30, 2016

Project Title: Environmental Fate of Antiepileptic Drugs and their Metabolites: Biodegradation, Complexation, and Photodegradation

Investigators	**Institutions**
Thomas Borch (PI)	Colorado State University
Yitzhak Hadar (co-PI)	The Hebrew University of Jerusalem
Tamara Polubesova (collaborating)	The Hebrew University of Jerusalem

Keywords: Sorption, white-rot fungi, solid-state fermentation, organic matter, agricultural soils, carbamazepine, lamotrigine, FT-ICR MS

Abbreviations: See attached list of abbreviations

Budget: IS: $175,000 US: $135,000 Total: $310,000

Thomas Borch

Signature
Principal Investigator (PI)

Marilyn Morrissey

Signature
Institution's Authorizing Official, Principal Institution

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Publication Summary (numbers)

	Joint IS/US authorship	US authors only	Israeli authors only	Total
Refereed (published, in press, accepted)	2	1	1	4
Submitted, in review, in preparation	2	0	0	2
Invited review papers	0	0	0	0
Book chapters	0	0	0	0
Books	0	0	0	0
Master theses	0	0	2	2
Ph.D. theses	0	1	1	2
Abstracts	1	0	3	4
Not refereed (proceedings, reports, etc.)	0	0	0	0

Postdoctoral Training: No postdocs received more than 50% of their funding by the grant.

Cooperation Summary (numbers)

	From US to Israel	From Israel to US	Together, elsewhere	Total
Short visits and meetings	1	0	1	2
Longer visits (sabbaticals)	0	0	0	0

Description Cooperation: Drs. Borch and Hadar (the two PIs) exchange information through e-mails. During the summer of 2014, Drs. Borch, Hadar and Polubesova met in Jerusalem to discuss the project's progress and expand collaborative efforts. In January, 2016, members of the research groups of Drs. Borch and Polubesova met at the National High Magnetic Field Laboratory in Tallahassee, Florida, USA, to receive instrument and software training and to analyze samples using FT-ICR MS. The groups collaborate by e-mail and teleconference regarding FT-ICR MS data processing and interpretation.

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Patent Summary (numbers)

	Israeli inventor only	US inventor only	Joint IS/US inventors	Total
Submitted	0	0	0	0
Issued (allowed)	0	0	0	0
Licensed	0	0	0	0

Unpublished Data

LTG Photodegradation

We completed experiments with an isotopologue of LTG to verify the proposed photoproduct structures contained in our published paper (Young et al. 2014) (Fig. 1).

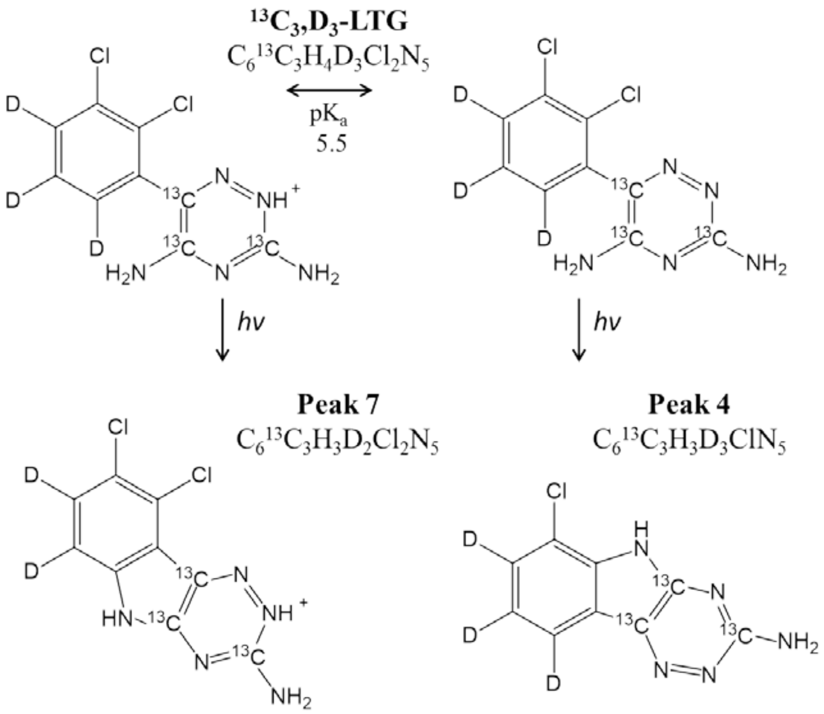


Figure 1. Verified structures for lamotrigine photoproduct peaks 4 and 7, as described in Young et al., 2014.

Because these photoproduct peaks appear to arise from photoinduced nucleophilic substitution reactions involving LTG's dichlorophenyl ring, additional experiments were conducted in the presence of various nucleophiles, including 4-pentenoic acid ($C_5H_8O_2$), and chloride (Cl^-) and bromide (Br^-) ions. The photodegradation rate of LTG was not enhanced in the presence of Br^- (Fig. 2), but bromine did substitute for chlorine on LTG's dichlorophenyl ring. The photodegradation rate of LTG was substantially enhanced in the presence of 4-pentenoic acid (Fig. 2), and several photoinduced nucleophilic substitution products were detected, including two $C_{14}H_{13}Cl_2N_5O_2$ peaks which appear to correspond to a lactone photosubstitution product. However, no trichlorinated photoproducts were detected. Together, these experiments suggest that LTG can undergo photoinduced nucleophilic substitution reactions in surface waters when suitable nucleophiles are present, possibly including nucleophilic functional groups in DOM.

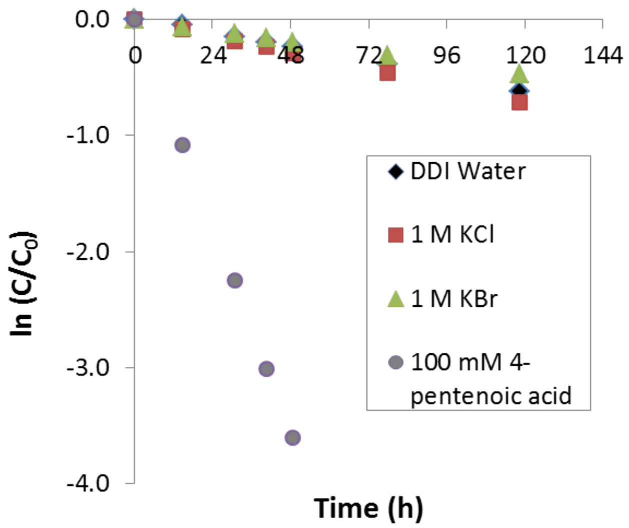


Figure 2. Photodegradation of lamotrigine in the presence of various nucleophiles.

Additional experiments were conducted to examine the photodegradation of LTG at pH 5.7 in the presence and absence of Suwannee River fulvic acid (SRFA). As in the case of 4-pentenoic acid, LTG's photodegradation was significantly enhanced in the presence of SRFA (Fig. 3). Many of the same photoproducts formed in both solutions, including peaks 4 and 7 described in Figure 2.

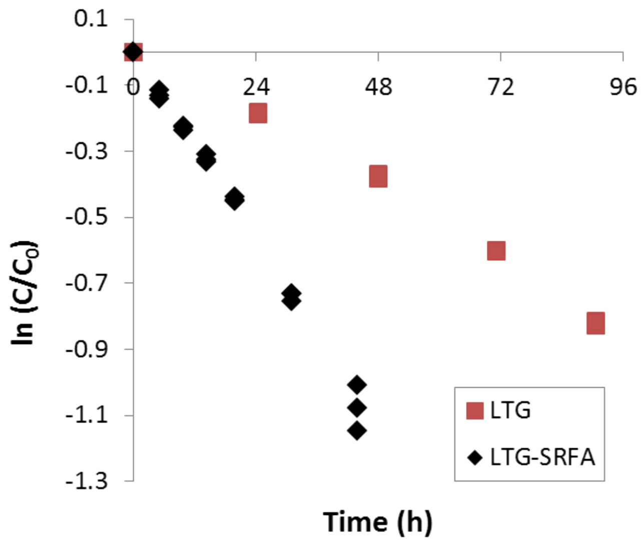


Figure 3. Photodegradation of lamotrigine at pH 5.7 in the presence and absence of Suwannee River fulvic acid (n=3).

LTG Biodegradation

We hypothesized that a prolonged exposure of agricultural soils to LTG via irrigation with treated wastewater would enable the development of a local microbial population that could degrade LTG. Several enrichment experiments were conducted with different soils type and growing conditions. External N source was not added to the medium for the purpose of using LTG as a sole N source, but LTG degradation was not observed. Similar findings were obtained when LTG was used as the sole C source. Even when the media was enriched with the anti-microbial agent Chloramphenicol or acidified (for the enrichment of the soils fungi population), LTG biodegradation was not observed. These findings support the assumption that LTG is highly resistant to biodegradation in the environment and particularly in soils.

We hypothesized that white rot fungi would be able to effectively degrade LTG through their variety of oxidizing enzymes. The fungi that were selected were: *Pleurotus ostreatus*, *Phanerochaete chrysosporium, Trametes versicolor, Phanerochaete sordida* sp.Y6 and *Trametes* sp. M23. Incubation of *P.ostreatus* with LTG in glucose peptone medium led to the highest LTG removal rate (70%). Incubation of *P.ostreatus* with LTG in Basidiomycete selective medium (BSM) exhibited a lower removal rate of only 15%. Incubation of both *T. versicolor* and *P. chrysosporium* with LTG in a glucose peptone medium led to small LTG removal rates of 14% and 5%, respectively. Two additional media were selected and defined as rich (PDB) or poor (low N). Incubation of *P. chrysosporium* and *P. sordida* SP.Y6 in both media led to low LTG removal (up to 22%). Incubation of *T. versicolor* and *T.* sp. M23 in the poor medium showed a higher removal rate (up to 33%), but still much lower than *P. ostreatus.*

Since *P. ostreatus* exhibited the highest efficiency in LTG removal, we studied the enzymatic mechanism involved in the process. In order to test the involvement of manganese peroxidase enzymes, the Mn^{+2} co-factor was added to the medium. Mn^{+2} amendment did not enhance LTG removal, suggesting that manganese-dependent enzymes are not involved in the process. When we added three different CYP-450 inhibitors to the glucose peptone medium, no inhibition was observed. Despite these findings we cannot conclude that CYP-450 enzymes are not involved in the process due to its many isoforms in the *P.ostreatus* genome, which are affected differently in response to different inhibitors.

LC-MS analysis was performed in order to identify the metabolites formed during incubation with *P.ostreatus*. For that, *P.ostreatus* was incubated in a diluted GP medium and with 100 mg/L LTG in order to dilute the background noise and improve the quality of the analysis. Some metabolites indicated the existence of reactions by CYP-450 and UDP-glucosyltransferase enzymes when sulfide derivatives and glycoside conjugation were found on the dichlorophenyl and triazine rings, respectively.

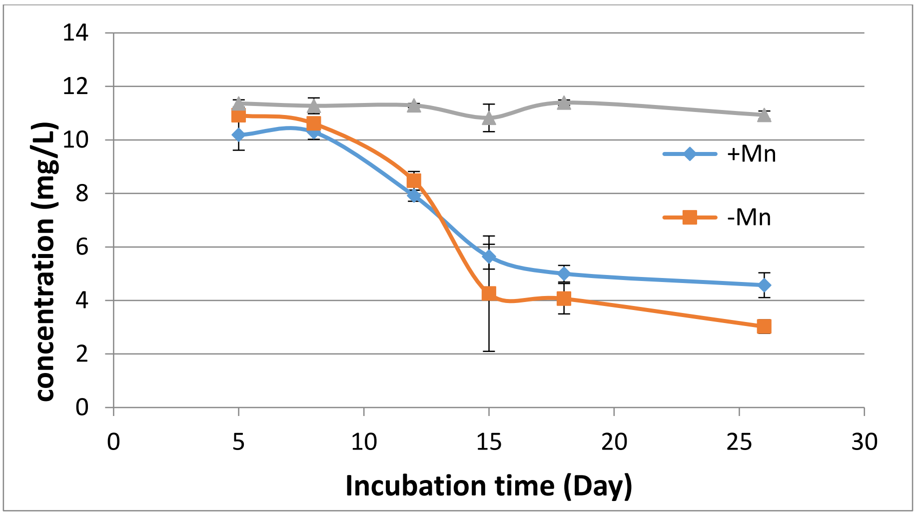


Figure 5. LTG degradation by *Pleurotus* grown in glucose peptone medium with and without Mn.

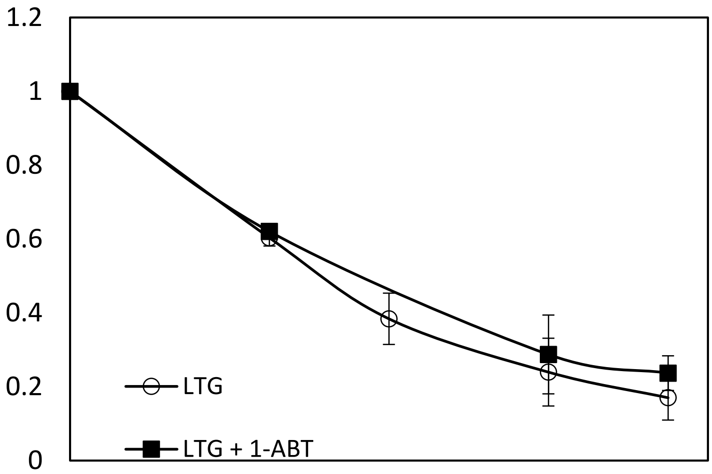


Figure 6. LTG degradation in the presence of 1-aminobenzotriazole, a CYP-450 inhibitor.

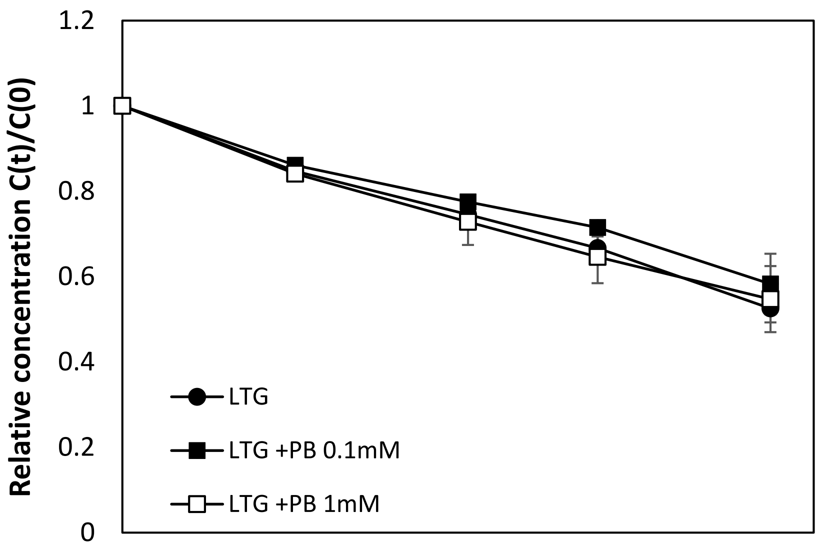


Figure 7. LTG degradation in the presence of piperonyl butoxide, a CYP-450 inhibitor.

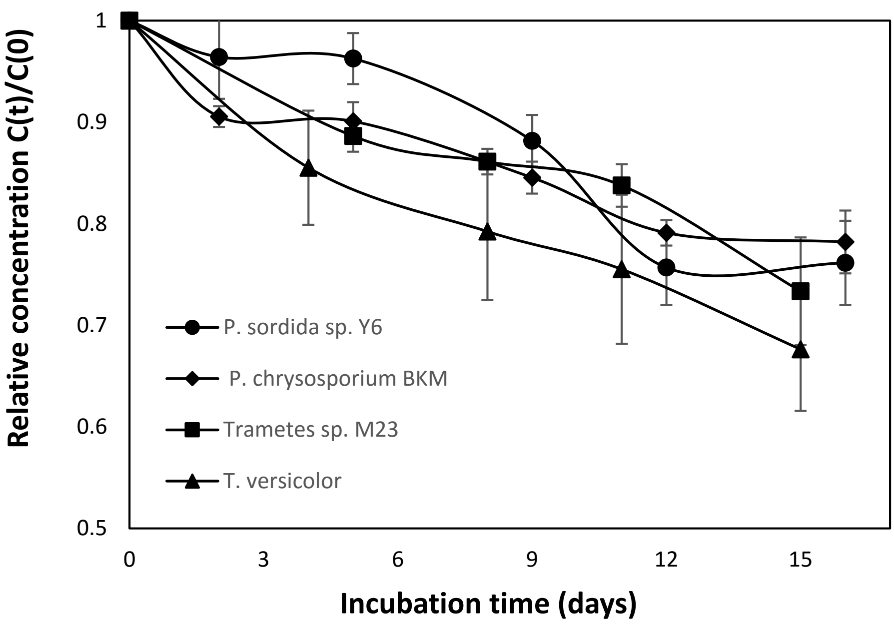
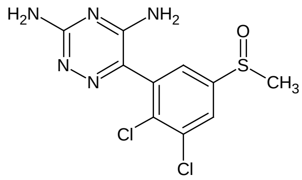
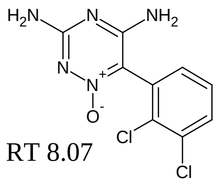
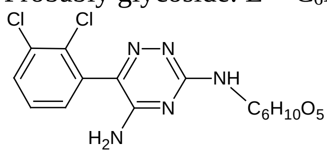
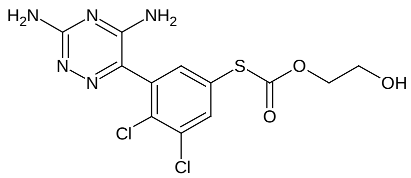
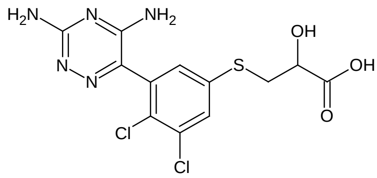
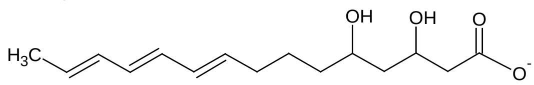


Figure 8. LTG degradation by other white rot fungi: *Trametes* versicolor, *Phanerochaete chrysosporium* BKM, *Phanerochaete sordida* sp. Y6, *Trametes* sp. M23.

Table 2. Tentative identification of metabolites in *Pleurotus* culture in the presence of LTG.

RT, min	Measured Mass	Atomic Composition	Proposed structure
7.44, 7.66	317.9976	$C_{10}H_{10}ON_5Cl_2S$	*L – [H] + –S(=O)–CH_3, MS/MS of 268.0 is needed. It can be for example: 
7.66	416.0516	$C_{15}H_{16}O_5N_5Cl_2$	MS/MS - NA
8.07, 9.1, 9.37 Neg. mode	272.0099 269.9952	$C_9H_8ON_5Cl_2$ $C_9H_6ON_5Cl_2$	 RT 8.07 Two other isomers are also N-oxides at aromatic ring.
8.19	418.0674	$C_{15}H_{18}O_5N_5Cl_2$	Probably glycoside: L + $C_6H_{10}O_5$. 
8.37	417.0291	$C_{14}H_{15}O_3N_6Cl_2S$	MS/MS - NA
8.42, 8.88 Neg. mode	376.0024 373.9883	$C_{12}H_{12}O_3N_5Cl_2S$ $C_{12}H_{10}O_3N_5Cl_2S$	 or 
8.78	499.0887	$C_{19}H_{21}O_6N_6Cl_2$	MS/MS - NA
9.28	256.0151	$C_9H_8N_5Cl_5$	Lamotrigine
11.38	302.0023	$C_{10}H_{10}N_5Cl_2S$	L – [H] + –S–CH_3
12.78, 13.16, 13.56	522.1658	$C_{24}H_{30}O_4N_5Cl_2$ ND in neg. mod.	L+ cyclic structure ($C_{14}H_{22}O_3$–CO–) or L+  of course, position of OH and double bonds is unknown.
12.75	538.1614	$C_{24}H_{30}O_5N_5Cl_2$	It is m/z 522 plus additional OH group.